European Journal of Pharmacology 702 (2013) 285–293

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Wound-healing effect of ginsenoside Rd from leaves of *Panax ginseng* via cyclic AMP-dependent protein kinase pathway

Wang-Kyun Kim [a,b,1], Seung-Yong Song [c,1], Won Keun Oh [d,1], Sireewan Kaewsuwan [a,b,e], Tien Lam Tran [d], Won-Serk Kim [f], Jong-Hyuk Sung [a,b,*]

[a] Department of Applied Bioscience, CHA University, #606-16, Yeoksam-dong, Kangnam-gu, Seoul 135-081, Republic of Korea
[b] Stem Cell Research Laboratory, CHA Stem Cell Institute, Seoul, Republic of Korea
[c] Department of Plastic and Reconstructive Surgery, Bundang CHA Medical Center, CHA University, Seongnam-si, Gyunggi-do, Republic of Korea
[d] BK21 Project Team, College of Pharmacy, Chosun University, Gwangju, Republic of Korea
[e] Marine Natural Products Research Unit (MNP), Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand
[f] Department of Dermatology, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 30 August 2012
Received in revised form
18 January 2013
Accepted 29 January 2013
Available online 8 February 2013

Keywords:
Ginsenoside Rd
Wound-healing
Cyclic AMP-dependent protein kinase
Keratinocyte progenitor cells
Human dermal fibroblasts

ABSTRACT

Panax ginseng is considered as one of the most valuable medicinal herbs in traditional medicine, and ginsenoside Rd is one of the main active ingredients in *P. ginseng* leaf. Although there is significant number of evidences implicated on the beneficial effects of the ginsenosides with diverse associated mechanisms, reports on the skin regeneration by the ginsenoside Rd are not sufficient. Therefore, we examined the mitogenic and protective effects of the ginsenoside Rd in the keratinocyte progenitor cells (KPCs) and human dermal fibroblasts (HDFs). Furthermore, the signaling pathways involved in the activation of KPCs and HDFs were investigated, and wound-healing effect is evaluated *in vivo* through animal wound models. We found that the ginsenoside Rd significantly increased the proliferation and migration level of KPCs and HDFs in a dose-dependent manner. Additionally, the cell survival was significantly increased in H_2O_2 treated KPCs. Moreover, the ginsenoside Rd effectively induced collagen type 1 and down-regulated matrix metalloprotinase-1 (MMP-1) in a dose-dependent manner. All of these beneficial effects are associated with an induction of intracellular cAMP levels and phosphorylated cAMP response element-binding protein expression in nucleus, which both attenuated by adenine 9-β-D-arabinofuranoside, an adenylate cyclase inhibitor. Application of the ginsenoside Rd to an excision wound in mice showed an effective healing process. As skin regeneration is mainly associated with the activation of HDFs and KPCs, *P. ginseng* leaf, an alternative source of the ginsenoside Rd, can be used as a natural source for skin regeneration.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Ginseng root (*Panax ginseng*) has been traditionally used in China, Korea and Japan to strengthen immune system, to provide nutrition, and to reduce fatigue (Kim et al., 2005). The major active constituent of ginseng is saponin, which contains a lot of identified ginsenosides (Cho et al., 2010; Paik et al., 1982). Each ginsenoside possesses individual pharmacological activities, and collectively has positive effects on human diseases, including atherosclerosis, liver dysfunction, cerebrovascular diseases, hypertension, post-menopausal disorder and cancers (Attele et al., 1999; Chen, 1996; Gillis, 1997; Nag et al., 2012). Of note, the purified ginsenosides or the extracts of ginseng root reportedly have beneficial effects on the damaged skin. For example, red ginseng root extract protected skin from acute UVB-irradiation (Kim et al., 2008), and the ginsenoside F_1, an enzymatically modified derivative of the ginsenoside Rg_1, protected HaCaT against UVB-induced apoptosis (Lee et al., 2003). *P. ginseng* root extract also promotes type I collagen synthesis in human dermal fibroblasts (HDF) via the smad activation pathway, and exhibits antioxidative activity against diphenyl-*p*-picrylhydrazyl treatment (Lee et al., 2007). In addition, ginsenoside Rb_1 promotes healing process of burn wound by enhancing angiogenesis (Kimura et al., 2006).

Although there are significant number of evidences implicated on the wound healing effects of the ginsenosides with diverse associated mechanisms, reports on the skin regeneration by the ginsenoside Rd or ginsenosides isolated from ginseng leaves

* Corresponding author at: Department of Applied Bioscience, CHA University, #606-16, Yeoksam-dong, Kangnam-gu, Seoul 135-081, Republic of Korea. Tel.: +82 2 3468 3491; fax: +82 2 3468 3373.
E-mail address: brian99@empal.com (J.-H. Sung).
[1] Equal contribution.

0014-2999/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejphar.2013.01.048

are not sufficient. Because a ginseng leaf is cheaper and easily acquired compared to a ginseng root, we isolated the active compounds from the Chinese ginseng leaf instead of ginseng root, and examined the novel wound-healing property. In the first place, we examined the mitogenic and protective effects of the ginsenoside Rd in the keratinocyte progenitor cells (KPCs) and HDFs. Furthermore, signaling pathways involved in the activation of KPCs and HDFs were investigated. Finally, the wound-healing effect of the ginsenoside Rd was tested through animal wound models.

2. Materials and methods

2.1. Materials and cell cultures

Antibodies against ERK, phosphorylated ERK, Akt, phosphorylated Akt, cAMP response element-binding protein (CREB), phosphorylated CREB, collagen type 1 and matrix metalloprotinase-1 (MMP-1) were obtained from Cell Signaling Technology (Danvers, MA). Antibody against β-actin, and secondary antibodies of horseradish peroxidase conjugated- anti-mouse IgG and anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Adenine 9-β-D-arabinofuranoside (Ara, an adenylate cyclase inhibitor) was purchased from Sigma (St. Louis, MO).

KPCs (CELLnTEC, Ontario) was maintained in Keratinocyte Growth Medium 2 (KGM2, PromoCell, Heidelberg, Germany) and HDFs (K-21947, Korean Cell Line Bank, Seoul, Korea) in DMEM containing 10% FBS (Gibco, Grand Island, NY), 1% Penicillin and Streptomycin (Gibco) at 37 °C in 5% CO_2 incubator (Kim et al., 2009; Sung et al., 2011).

2.2. Ginsenoside Rd isolation

2.2.1. General experimental procedures

NMR spectra were obtained from a Varian Inova 500 MHz spectrometer with TMS as an internal standard used at Korea Basic Science Institute (KBSI, Gwangju Center, Korea). The FAB and HRFAB-MS data were obtained from a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. Silica gels (Merck, 63–200 μm particle size; Merck, 40–63 μm particle size) and Sephadex LH-20 were used for column chromatography. TLC was carried out using silica gel $60F_{254}$ and RP-$18F_{254}$ plates. HPLC was carried out using a Gilson system with a UV detector and an Optima Pak C_{18} column (10×250 mm^2, 10 μm particle size, RS Tech, Korea). All solvents used for the extraction and isolation were analytical grades.

2.2.2. Plant material

P. ginseng leaves were purchased from Mergens Co. in Suwon City, Gyeonggi-do, Korea, and authenticated by Professor W.K. Oh at Chosun University.

2.2.3. Extraction and isolation

Ginsenoside Rd was extracted from ginseng leaves with 70% EtOH (6 L/sonication) at 60 °C via sonication for three times (2 h/sonication). And then, the resulting extract was concentrated into a residue (950 g). This crude extract was then dissolved in H_2O, and fractioned by a HP-20 column (20×50 cm^2), using a stepwise gradient solvents of 25% methanol, 75% methanol and acetone. After, the concentrated fraction (180 g) was chromatographed on a silica gel column (20×60 cm^2; 63–200 μm particle size), and eluted with ethyl acetate/methanol (10:0, 10:1 to 0:1 each 6.0 L) to yield seven fractions (F1: 13.3 g; F2: 35.3 g; F3: 22.8 g; F4: 41.6 g; F5: 32.2 g; F6: 13.0 g; F7: 24.5 g). Fraction F5 was chromatographed over a Sephadex LH-20 column (7×30 cm^2), using methanol as the eluting solvent, to give seven sub-fractions (F5.1–F5.7). Sub-fraction F5.6 was separated by RP-C18 (6×50 cm^2) with a stepwise gradient of solvent, methanol/water (1:3–3:0), to isolate ginsenoside Rd (1.250 g).

2.3. Proliferation assay

KPCs (4×10^4/well) and HDFs (3×10^4/well) were seeded in 6-well plates in the Keratinocyte Growth Medium 2 (KGM2) and DMEM with 10% FBS. The media were then changed to the Keratinocyte Basal Medium 2 (KBM2) or DMEM overnight. After starvation, cells were treated with various fractions (1 μg/ml) or various concentrations of the ginsenoside Rd. Then, cells were incubated in KGM2 or complete medium for 48 h at 37 °C in 5% CO_2 atmosphere, and the MTT assay was performed. MTT solution (5 mg/ml in PBS) was added to each well at 1/20 volume of the media. Cells were incubated at 37 °C for 2 h, and the supernatant were removed. Dimethyl sulfoxide (DMSO) was then added in order to dissolve the formazan crystals, and the absorbance was measured at 595 nm using an ELISA reader (TECAN, Grodig, Austria).

2.4. Migration assay

For the measurement of cell migration, confluent KPCs and HDFs were starved in serum-free media for 24 h. Then, cells were pre-treated with various concentrations of the ginsenoside Rd for 2 h. The pre-treated cells were then wounded with a plastic micropipette tip having a large orifice, and further incubated with the same concentrations of ginsenoside Rd in KBM2 with 0.1% supplement or DMEM with 0.1% FBS. Photographs of wound area were taken at 48 h after ginsenoside Rd treatment in KPCs and 72 h in HDFs by phase-contrast microscopy (CKX41SF, Olympus, Tokyo, Japan). To determine wound closure, four randomly selected points along each wound were marked, and the horizontal distance of migrating cell from the initial wound was measured.

2.5. Cell survival assay

Effect of ginsenoside Rd on KPCs survival was achieved by assessing the cell survival using MTT assay and by fractioning cells with sub-G1 phase through flow cytometry. KPCs were seeded in 6-well plate (1×10^5/well) or 100 mm dish (8×10^5/well), incubated and allowed to grow to 80% confluence. Cells were then pretreated with various concentrations of the ginsenoside Rd for 2 h in KBM2, and the media were changed to 0.5 mM H_2O_2 with the same concentration of ginsenoside Rd in KBM2. The cells were incubated for 24 h before the analysis.

KPCs treated with 10 μM ginsenoside Rd was used for flow cytometry. KPCs were washed twice with cold PBS, harvested, and permeabilized with cold 70% EtOH before the analysis. The cells were then washed with PBS, containing 0.1% BSA and 0.2% Tween 20. Cellular DNA was stained with 50 μg/ml propidium iodide (PI)-treated RNase (100 μg/ml). The distribution of cell cycle phases with different DNA contents was examined in a FACScan flow cytometer (Becton-Dickinson, Frankinson Lakes, NJ).

For fluorescence microscope detection, KPCs were fixed by 1% paraformaldehyde in PBS and placed in an ice bath for 30 min. In addition, 4′,6-diamidino-2-phenylindole (DAPI, 1:1000) (Roche, Indianapolis, Indiana) was counterstained. Stained cells were examined, and photographed by a fluorescence microscope (ECLIPSE E600, Nikon).

2.6. cAMP immunoassay

To determine the cAMP levels in KPCs, starved KPCs were treated with various concentrations of the ginsenoside Rd for 1 h. After washing with PBS and trypsinization, the cells were collected for further lysis by using 0.1 M HCl. The cAMP levels were then analyzed from the supernatant using the cyclic AMP EIA Kit (Cayman, Ann Arbor, MI) according to the standard protocol. Developed color was measured at a wavelength between 405 and 420 nm.

2.7. Immunofluorescence of pCREB

KSCs were cultured directly on glass cover-slips and treated with ginsenoside Rd for 1 h. Then, KPCs were fixed with 3.5% paraformaldehyde for 10 min at room temperature, after which they were permeabilized with 100% methanol for 10 min. To evaluate phosphorylation of CREB, cells were incubated with antibody against phospho-CREB overnight (a 1:1000 dilution in PBS). Then, cells were washed with PBS and incubated with a 1:500 dilution of secondary fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG antibody for 1 h at room temperature. Finally, the nuclei were stained with 1 mg/ml of 4′,6-diamidino-2-phenylindole (DAPI) and fluorescence signals were analyzed by fluorescence microcopy (ECLIPSE E600, Nikon).

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA of HDFs, treated with various concentrations of the ginsenoside Rd for 24 h, was extracted with Trizol reagent followed by a reverse transcription to cDNA. The following oligonucleotides were used as primers: collagen type I (5′-TAGGGTCTAGACATGTTCAGCTTTGT-3′ and 5′-GTGATTGGTGGGATGTCTTCGT-3′), MMP-1 (5′-AGATGTGGAGTGCCTGATGT-3′ and 5′-AGCTAGGGTACATCAAAGCC-3′), and the control GAPDH (5′-CGAGATCCCTCCAAAATCAA-3′ and 5′-TGTGGTCATGAGTCCTCCCA-3′). The PCR was carried out in a total volume of 30 μl for PCR amplification of cDNA, reverse-transcribed from the total RNA. After initial denaturation at 95 °C for 5 min, amplification was performed in 35 cycles 30 s at 95 °C, 20 s at 54 °C, and 30 s at 72 °C and followed by a final extension at 72 °C for another 10 min. GAPDH mRNA level was used for sample standardization.

2.9. Western blotting analysis

Cells (2×10^5) were plated into 60 mm dish, cultured overnight, and starved in serum free media for 24 h. Cells were then exposed to various concentrations of the ginsenoside Rd for 1 h for measuring phosphorylation of Akt, ERK1/2 and CREB, and 24 h for measuring collagen and MMP1. Treated cells were lysed with 1 × RIPA buffer (50 mM Tris–HCl, 0.15 M NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.4, 1% SDS, 50 mM NaF, 1 mM Na_3VO_4, 5 mM Dithiothreitol, 1 mg/ml Leupeptin and 20 mg/ml 1 mM PMSF). The lysates were then centrifuged at 13000 rpm for 15 min at 4 °C, and then the supernatants were collected for immunoblotting. The total protein in the sample was determined by Bardford assay (Bio-Rad, Hercules, CA) using ELISA reader. Each of the 30 μg sample proteins was separated into 10–12% SDS–polyacrylamide gel by

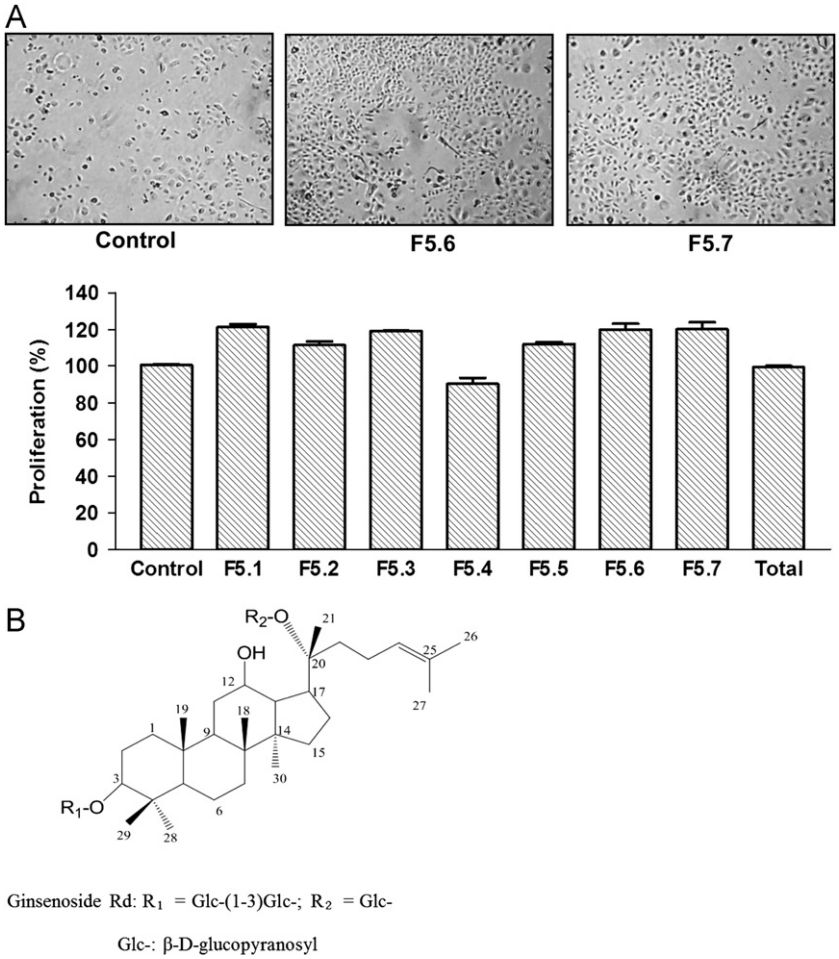

Fig. 1. Cell proliferating effect of subfractions of ginseng leaf and the structure of the ginsenoside Rd. KPC proliferation was measured by MTT assay after treatment of 1 μg/ml concentration of seven subfractions obtained from ginseng leaf (A). Chemical structure of ginsenoside Rd (B).

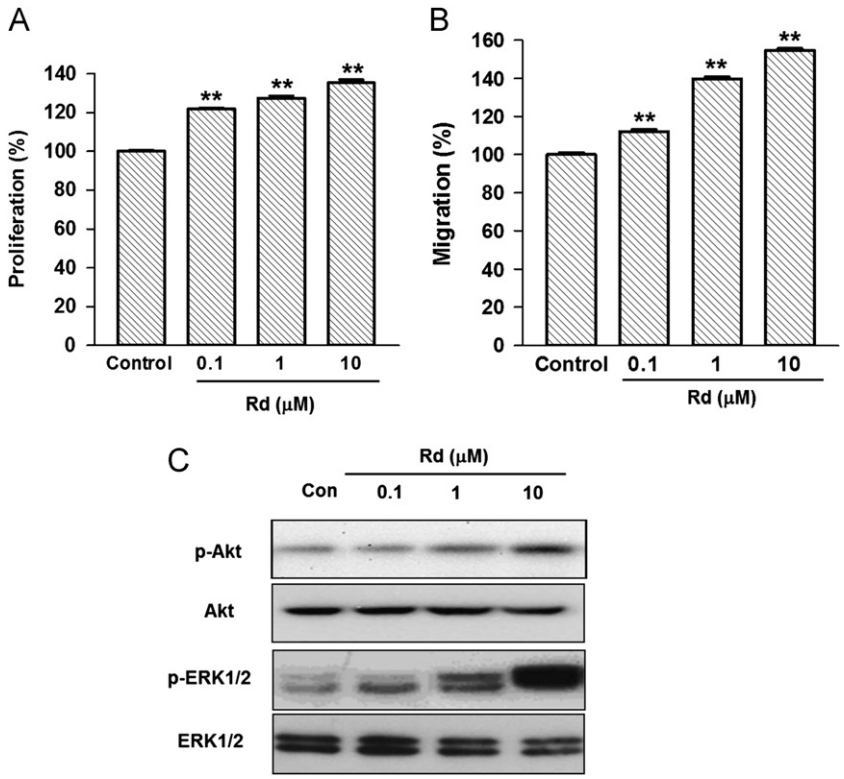

Fig. 2. Effects of the ginsenoside Rd on proliferation and migration of KPCs. Changes of KPC proliferation treated with various concentrations of the ginsenoside Rd (at 48 h) was monitored using MTT assay (A), and KPC migration (at 48 h) was photographed using phase-contrast microscopy (B). The levels of phosphorylated Akt and ERK were determined by Western blot at 1 h after ginsenoside Rd treatment (C). Ginsenoside Rd significantly increased KPC proliferation, migration and the levels of phosphorylated Akt and ERK in a dose-dependent manner. The results were represented with means ± S.E.M ($n=3$). $^{**}P<0.01$ compared to the control.

electrophoresis. Proteins were then transferred to the PVDF membranes, which were subsequently blocked with 5% skim milk in TBS buffer containing 0.1% Tween 20, before incubating it with anti-phosphorylated ERK1/2 (1:2000), anti-total ERK1/2 (1:4000), anti-phosphorylated Akt (1:1000) and anti-total Akt(1:4000), anti-phosphorylated CREB(1:1000), CREB(1:1000), MMP-1(1:1000), anti-collagen (1:1000) or anti-β-actin antibodies (1:1000). The membranes were then washed, and incubated with horseradish peroxidase conjugated-anti-mouse IgG or anti-rabbit IgG antibodies. Blots were reacted with immobilon Western reagent (ECL; Millipore, Billerica, MA), and exposed to X-ray film.

2.10. Inhibition studies for PKA pathway

To verify the involvement of protein kinase A (PKA) pathway in ginsenoside Rd-induced stimulation of KPCs and HDFs, pharmacological inhibition study was performed. Both KPCs and HDFs were treated with 10 μM ginsenoside Rd in the presence or absence of various concentrations of Ara. The proliferation assay, migration assay, Western blotting assay and cAMP immunoassay were then re-performed.

2.11. Wound healing in vivo

As a laser burn wound model and an excision wound model, 2 month-old female hairless mice were assigned. Before the wound induction, food was withheld for atleast 6 h prior to anesthesia with subcutaneous injection of Zoletil (30 mg/kg) and Rompun (10 mg/kg) mixture. Experimental protocols involving the mice used in this study were reviewed by the Animal Care and Use Committee in CHA University according to the NIH guidelines (NIH publication number 85–23, revised 1985) of Principles of Laboratory Animal Care.

For excision wound model, the back skin of a mouse was punched in full thickness with dermal punch (8 mm diameter, Kai Industries co. Ltd., Seki City, Japan). A wound placed in this area cannot be reached by the mouse, and therefore was prevented from self-licking. Either control (100 μl, matrigel solution) or 10 μM ginsenoside Rd in matrix gel solution were applied to the wounded control and experimental mice, respectively, and treated every 2 days until day 10. The treated-wounds were then bandaged the wound area with transparent Tegaderm® (3 M Health Care, St. Paul, MN). The wounds from individual mouse were digitally photographed, and measured the area every 3 days, from the day of wounding.

For the laser burn wound model, a superficial degree thermal injury was created by a CO_2 fractional laser (Lutronic Inc., Seoul, Korea) on the mice dorsal midline. The laser controlled depth of the thermal damage, limiting the depth of wound to epidermal area. Control (Vaseline vehicle), 0.1% and 0.5% ginsenoside (w/v) were applied to the superficial wounds, and bandaged the wound area with transparent Tegaderm® (3M Health Care, St. Paul, MN). The wounds from each individual mouse were digitally photographed, and measured the area of wound every 2 days excluding the weekend, from the day of wounding to day 10.

2.12. Statistical analysis

All data are presented as mean ± SE, and statistical difference between groups were assessed by Student's t-test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Characterization of ginsenoside Rd

Fractioned ginsenoside Rd was dried to get a white powder. Ginsenoside Rd was characterized by mass and NMR spectroscopy. FAB-MS (m/z): calcd for $C_{48}H_{82}O_{18}$ $[M+H]^+$: 947.55, found: 947.70;

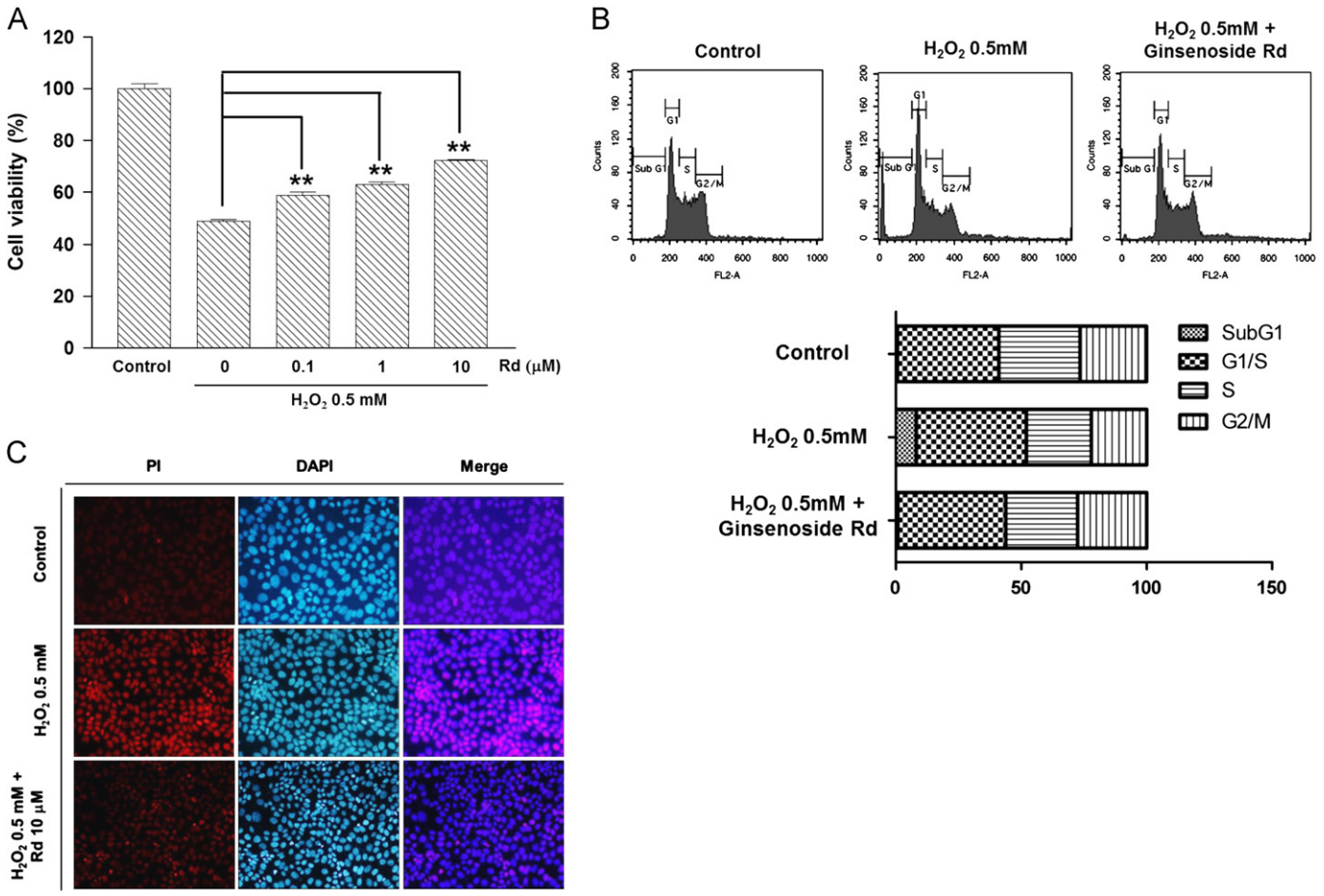

Fig. 3. Protective effects of the ginsenoside Rd on H_2O_2-exposed KPCs. Cell viability of KPCs, treated with various concentrations of the ginsenoside Rd, was measured in the presence of 0.5 mM H_2O_2 using MTT assay (A). The cell cycle phases of KPCs incubated with 0.5 mM H_2O_2 and 10 μm ginsenoside Rd were determined by flow cytometry (B) and immunostaining (C). Ginsenoside Rd significantly attenuated the cell survival reduced by H_2O_2 treatment in a concentration-dependent manner. The results were represented with means ± S.E.M ($n=3$). $^{**}P<0.01$ compared to the control.

^{1}H-NMR (C_5D_5N, 500 MHz, Supplemental Fig. S1A): δ_H (ppm) 0.77 (s, CH_3-30), 0.92 (s, CH_3-30), 0.92 (s, CH_3-18), 1.05 (s, CH_3-29), 1.22 (s, CH_3-21), 1.57 (s, CH_3-26), 1.57 (s, CH_3-27), 1.57 (s, CH_3-28), 3.21 (m, H-3), 3.44 (m, H-12), 4.84 (d, J=7.5 Hz, H-1 of Glc I), 5.10 (d, J=7.5 Hz, H-1 of Glc III), 5.21 (dt, J=6.5 Hz, H-24), 5.27 (d, J=7.5 Hz, H-1 of Glc II); ^{13}C-NMR (C_5D_5N, 125 MHz, supplemental Fig. S1B): δ_C (1): 39.4 (2) 26.8 (3) 89.2 (4) 39.8 (5) 56.6 (6) 18.6 (7) 35.3 (8) 40.2 (9) 50.4 (10) 36.2(11) 30.9 (12) 70.5 (13) 49.6 (14) 51.6 (15) 30.9 (16) 26.8 (17) 51.9 (18) 17.5 (19) 16.5 (20) 83.5 (21) 22.7 (22) 36.2 (23) 23.4 (24) 126.1 (25) 131.2 (26) 26.0 (27) 16.8 (28) 28.3 (29) 18.0 (30) 16.2. Glc I: (1′) 105.2 (2′) 83.4 (3′) 78.2 (4′) 71.6 (5′) 78.1 (6′) 62.9 Glc II: (1″) 106.0 (2″) 77.1 (3″) 79.2 (4″) 71.7 (5″) 78.4 (6″) 62.8. Glc III: (1‴) 98.2 (2‴) 75.0 (3‴) 78.3 (4‴) 71.6 (5‴) 78.2 (6‴) 62.7.

3.2. Ginsenoside Rd increases the proliferation and migration of KPCs

We sub-fractionated seven fractions (F5.1–F5.7) from leaf of *P. ginseng*, and tested the proliferating effect on KPCs. F5.6 and F5.7 showed the positive effect on KPC proliferation (at 1 μg/ml, Fig. 1A). Then, we found the major compounds from these fractions and isolated ginsenoside Rd (Fig. 1B). As shown in Fig. 2A, the ginsenoside Rd treatments significantly increased the KPC proliferation compared to the non-treatment group in a dose-dependent manner. Likewise, ginsenoside Rd treatment for 48 h significantly increased the KPC migration, and the migration level was proportional to the ginsenoside Rd concentration (Fig. 2B). To investigate the mitogenic signals by which the ginsenoside Rd controls the proliferation and migration of KPCs, we measured the phosphorylation of ERK1/2 and Akt, which are the important mitogenic factors in many cell types (Impey et al., 1999; Peyssonnaux and Eychène, 2001; Wada et al., 2006). Strong increase of the phosphorylated ERK and Akt was observed in a dose-dependent manner (Fig. 2C).

3.3. Ginsenoside Rd increases KPCs survival from free radicals

After H_2O_2 treatment, KPCs began to detach and they loss normal morphology. However, cell detachment was less severe in ginsenoside Rd-treated KPCs (data not shown). As shown in Fig. 3A, viable cells were progressively reduced after 0.5 mM H_2O_2 treatment, whereas viable cells were significantly enhanced in KPCs treated with 0.1–10 μM ginsenoside Rd.

In addition, the cell cycle analysis by the flow cytometry supported an idea that the ginsenoside Rd exhibited anti-apoptosis effect against H_2O_2, since the population of sub-G1 cells was dramatically diminished after ginsenoside Rd treatment (from 7.93% to 0.80%, Fig. 3B). In a fluorescence staining, treatment of KPCs with 10 μM of ginsenoside significantly reduced the PI-stained cells (Fig. 3C).

3.4. cAMP and PKA pathway are involved in KPC stimulation

PKA signaling pathway reportedly plays a key role in pharmacological action of ginsenoside (Chung et al., 1998; Stork and

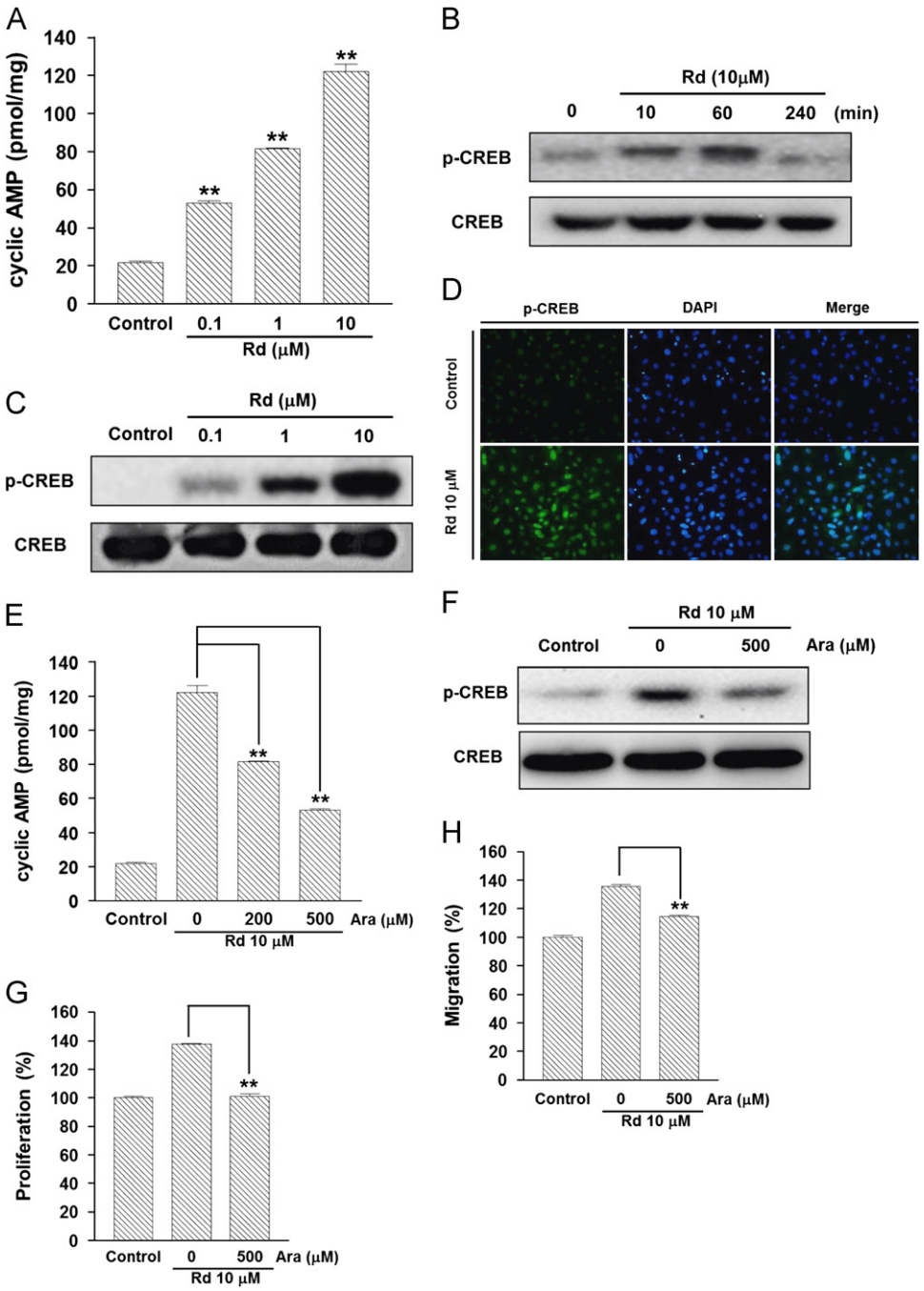

Fig. 4. Involvement of PKA pathway in ginsenoside Rd-induced stimulation of KPCs. cAMP levels was measured using cyclic AMP ELISA Kit 1 h after ginsenoside Rd treatment (A), Temporal profile of phosphorylated CREB proteins level was determined using Western blot (B) and CREB phosphorylation increased in a dose-dependent manner at 1 h after ginsenoside Rd treatment (C). Phosphorylated CREB matched with DAPI, and fluorescence signal was increased after ginsenoside Rd treatment (D). Ginsenoside Rd-induced cAMP level was attenuated by 9-β-D-arabinofuranoside (Ara, an adenylate cyclase inhibitor) treatment (E). In addition, Ara significantly inhibited the ginsenoside Rd-induced CREB phosphorylation (F), proliferation (G), and migration (H) of KPCs. The results were represented with means ± S.E.M ($n=3$). $^{**}P<0.01$ compared with the control.

Schmitt, 2002; Xue et al., 2006; Yuan et al., 2012). Therefore, we examined whether or not PKA pathway is involved in skin regeneration by ginsenoside Rd. In the first place, cAMP level was significantly increased by the ginsenoside Rd treatment (at 1 h) in a dose-dependent manner (Fig. 4A). Therefore, we detected the phosphorylated CREB protein, one of the transcription factors regulated by PKA pathway after the ginsenoside Rd treatment. Phosphorylated CREB was measured using Western blotting, and it was significantly increased in a time dependent manner (Fig. 4B) and was high at 1 h after ginsenoside Rd treatment. In addition, ginsenoside Rd treatment increased the CREB phosphorylation in a dose-dependent manner (Fig. 4C). In addition, the ginsenoside Rd treatment increased the fluorescence signal of the phosphorylated CREB in immunofluorescence (Fig. 4D).

To demonstrate whether or not ginsenoside Rd mediates the proliferation and migration through the activation of PKA pathway, we exposed KPCs to the Ara, which is an adenylate cyclase inhibitor, in the presence of 10 μM ginsenoside Rd. The data showed that the cAMP levels were decreased by 200 and 500 μM Ara treatment (Fig. 4E). Moreover, the level of phosphorylated CREB was decreased by 500 μM Ara treatment (Fig. 4F). Likewise, proliferation and migration of KPCs were significantly reduced by

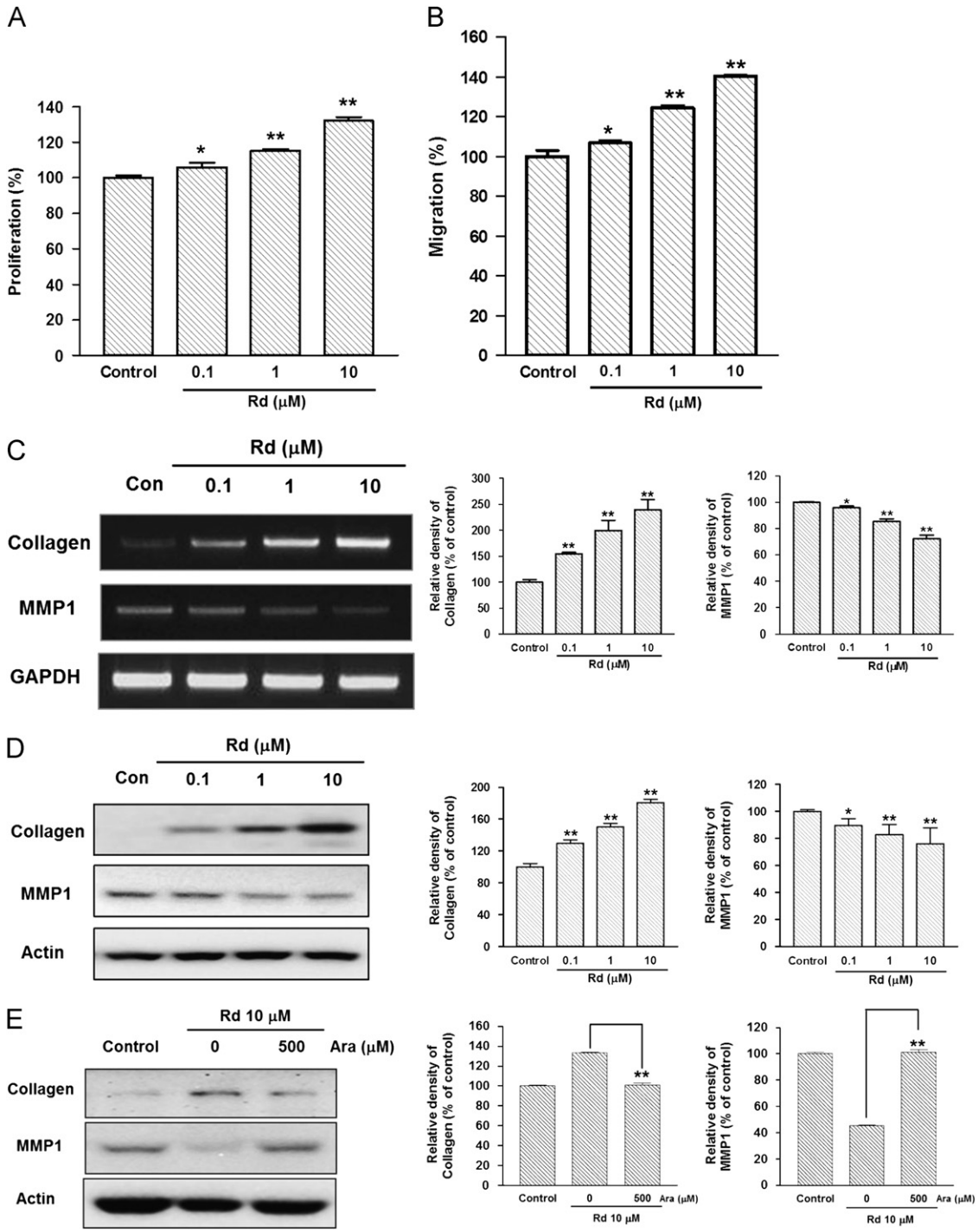

Fig. 5. Effects of the ginsenoside Rd on the proliferation, migration, and extracellular matrix protein production of HDFs. Changes of HDFs proliferation treated with various concentrations of the ginsenoside Rd (at 48 h) were monitored using MTT assay (A), and HDFs migration (at 72 h) was photographed using phase-contrast microscopy (B). Ginsenoside Rd significantly increased HDFs proliferation and migration in a dose-dependent manner. The mRNA expression of type I collagen and MMP1 in HDFs was measured by RT-PCR 24 h after ginsenoside Rd treatment (C), and the protein levels of type I collagen and MMP-1 were detected by using Western blotting (D). Ginsenoside Rd at 0.1–10 μM significantly increased collagen type I protein, and decreased MMP-1 protein in HDFs in a concentration-dependent manner. However, the reverse effects were observed with Ara treatment (E). The results were represented with means ± S.E.M ($n=3$). $^{*}P<0.05$; $^{**}P<0.01$ compared with the control.

500 μM Ara treatment (Fig. 4G and H). Collectively, these results indicate that the ginsenoside Rd induces the PKA pathways, stimulating the proliferation and migration of KPCs.

3.5. Ginsenoside Rd increases the proliferation and migration of HDFs

The stimulatory effects of the ginsenoside Rd on HDFs were also investigated. The HDF proliferation was significantly increased in 1 and 10 μM ginsenoside Rd treatment (Fig. 5A). In addition, ginsenoside Rd significantly increased the migration of HDFs (Fig. 5B).

3.6. Ginsenoside Rd increases ECM protein synthesis in HDFs

Beside the proliferation and migration of HDFs, 0.1–10 μM ginsenoside Rd increased the mRNA expression of collagen type 1 in HDFs (Fig. 5C). However, ginsenoside Rd treatment reduced

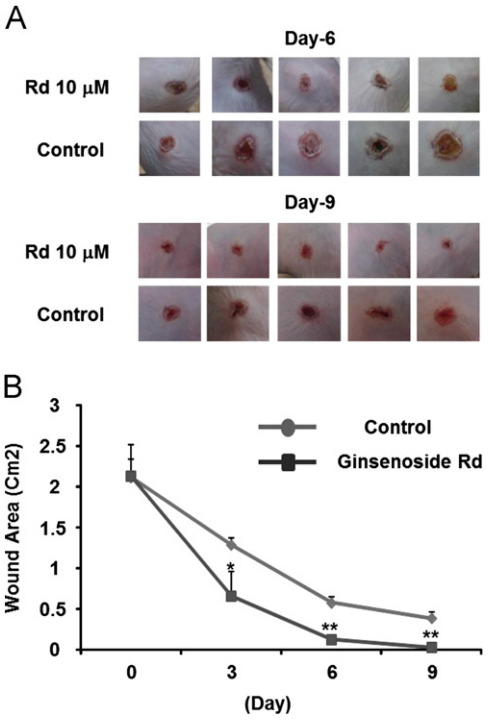

Fig. 6. Effects of the ginsenoside Rd on excision wound in mice. After the excision wound was made by dermal punch (8 mm diameter), either control (matrigel solution) or 10 μM ginsenoside Rd in matrix gel solution was applied to the wound surface. The size of the wound was measured every 3 days from the day of wounding. The wound area treated with 10 μM ginsenoside Rd was smaller than the area treated with control according to the data observed at day 6 and 9 (A). The ginsenoside Rd-treated wounds were significantly smaller than the wounds treated with control matrigel solution in a time-dependent healing consequence (B). The results were represented with means ± S.E.M ($n=5$). *$P<0.05$; **$P<0.01$ compared to the control.

MMP-1 mRNA level. Similarly, Western blot analysis revealed an increase in collagen protein level and a decrease in MMP-1 protein level in HDFs after 0.1–10 μM ginsenoside Rd treatment (Fig. 5D). On the other hand, reverse effects were observed in Ara treatment (Fig. 5E). This finding suggests an involvement of PKA pathway in the induction of collagen type 1 and the reduction of MMP-1 after ginsenoside Rd treatment.

3.7. Effect of ginsenoside Rd on in vivo wound healing

To determine whether or not ginsenoside Rd has a therapeutic effect on wound healing, Vaseline-based 0.1% or 0.5% ginsenoside Rd was applied to the epidermal wound induced by a laser burn on the back of mice, and the wound size was measured. The observation on the wound size, observed from 2 to 10 days, showed that the ginsenoside Rd significantly decreased the wound size (supplemental Fig. S2A and B). In addition, the excision wound was created and wound-healing effect of the ginsenoside Rd was measured. As shown in Fig. 6A and B, the matrigel solution containing the ginsenoside Rd (10 μM concentration) demonstrated the more potent healing outcome in the excision wound animal model compared to the control mice. These results indicate that the ginsenoside Rd has wound healing property *in vivo*.

4. Discussion

As described in introduction section, there are a lot of evidences implicating on the beneficial effects of the ginsenosides in diverse tissues, however, the wound-healing effect of the ginsenoside Rd and its mechanism of action has not yet been reported. The ginsenoside Rd remarkably increased the proliferation and migration of KPCs and HDFs, which collectively accelerated wound healing in laser and excision wounds. The ginsenoside Rd mediated wound healing via generation of cAMP and increase of CREB phosphorylation. This is the first indication that the ginsenoside Rd has wound-healing effect through the PKA pathway.

Ginsenosides are the steroid-like molecules that have a dammarane steroid nucleus with 17 carbon atoms and different sugar moieties attached (Nag et al., 2012). It has been suggested that the ginsenosides are permeable to cell membrane due to their steroid-like structures, which justify their ability to regenerate skin. Previously, the ginsenoside Rb_1 and Rb_2 were reported to have wound-healing effects (Kanzaki et al., 1998; Kimura et al., 2006). Ginsenosides Rb_1, Rb_2 and Rd belong to triterpenoid saponin compound that carries the comparable structures. Interestingly, the pharmaceutically active ginsenoside Rd can be produced by hydrolyzing sugar moieties from the ginsenoside Rb_1 through microorganisms (Chen et al., 2006; Chi and Ji, 2005; Dong et al., 2003; Kim et al., 2005) and enzymatic production such as β-glucosidase from *Thermus caldophilus* (Son et al., 2008). It is reasonable that the ginsenosides Rb and Rd contain the same core structure that is necessary in wound healing activity. Chemical structure of sugar moiety attached to the 20-position of the triterpene dammarane might contribute to wound healing effect of ginsenosides.

Our investigation included a key mediator and a signal pathway involved in the stimulation of KPCs and HDFs by the ginsenoside Rd. The cAMP level increased when the ginsenoside Rd was supplemented. On the contrary, the ginsenoside Rd-induced cAMP level was greatly reduced by the Ara treatment. Similarly, phosphorylated CREB expression was significantly increased by the ginsenoside Rd-treated KPCs, whereas the Ara treatment reduced the phosphorylated CREB level. Ginsenoside Rd-induced KPCs proliferation and migration were attenuated by the Ara treatment, confirming the involvement of cAMP generation in KPCs stimulation by ginsenoside Rd treatment. In addition, the Ara treatment of HDFs resulted in the down-regulation of collagen and up-regulation of MMP-1 expressions. Collectively, these results indicate that the ginsenoside Rd plays a key role in skin regeneration through PKA pathway.

In addition to the *in vitro* evidences suggesting the stimulation of KPCs and HDFs by the PKA pathway, the ginsenoside Rd significantly decreased the wound sizes and accelerated the healing process for both laser burn and excision wounds *in vivo*. Therefore, *P. ginseng* leaf, an alternative source of the ginsenoside Rd, can be used as a natural source for skin regeneration.

Acknowledgments

English is kindly edited by Soo-Min Kim (Edmonton, Alberta, CA) and Samivel Ramachandran (CHA University). This research was supported grants (2011–0019636 and 2011–0007751) from the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology. The authors declare no competing financial interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2013.01.048.

References

Attele, A.S., Wu, J.A., Yuan, C.S., 1999. Ginseng pharmacology multiple constituents and multiple actions. Biochem. Pharmacol. 58, 1685–1693.

Chen, L.Q., Kim, M.K., Lee, W., Lee, Y.J., Yang, D.C., 2006. Conversion of major ginsenoside Rb_1 to ginsenoside F_2 by *Caulobacter leidyia*. Biotechol. Lett. 28, 1121–1127.

Chen, X., 1996. Cardiovascular protection by ginsenosides and their nitric oxide releasing action. Clin. Exp. Pharmacol. Physiol. 23, 728–732.

Chi, H., Ji, G.E., 2005. Transformation of ginsenosides Rb_1 and Re from *Panax ginseng* by food microorganisms. Biotechnol. Lett. 27, 765–771.

Cho, J.G., Lee, M.K., Lee, J.W., Park, H.J., Lee, D.A., Lee, Y.H., Yang, D.C., Baek, N.I., 2010. Physicochemical characterization and NMR assignment of ginsenosides Rb_1, Rb_2, Rc and Rd isolated from *Panax ginseng*. J. Gingseng Res. 34, 113–121.

Chung, E., Lee, K.Y., Lee, Y.J., Lee, Y.H., Lee, S.K., 1998. Ginsenoside Rg_1 down-regulates glucocorticoid receptor and displays synergistic effects with cAMP. Steroids 63, 421–424.

Dong, A., Ye, M., Guo, H., Zheng, J., Guo, D., 2003. Microbial transformation of ginsenoside Rb_1 by *Rhizopus stolonifer* and *Curcularia lunata*. Biotechnol. Lett. 25, 339–344.

Gillis, C.N., 1997. *Panax ginseng* pharmacology: a nitric oxide link? Biochem. Pharmacol. 54, 1–8.

Impey, S., Obrietan, K., Storm, D.R., 1999. Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. Neuron 23, 11–14.

Kanzaki, T., Morisaki, N., Shiina, R., Saito, Y., 1998. Role of transforming growth factor-b pathway in the mechanism of wound healing by saponin from Ginseng Radix rubra. Br. J. Pharmacol. 125, 255–262.

Kim, M.K., Lee, J.W., Yang, D.C., 2005. Microbial conversion of major ginsenoside Rb_1 to pharmaceutically active minor ginsenoside Rd. J. Microbiol. 43, 456–462.

Kim, W.S., Park, P.B., Kim, S.H., Sung, J.H., H.K., 2009. Antiwrinkle effect of adipose-derived stem cell: activation of dermal fibroblast by secretory factors. J. Dermatol. Sci. 53, 96–102.

Kim, Y.G., Sumiyoski, M., Sakanaka, M., Kimura, Y., 2008. Effects of red ginseng extract on ultraviolet B-irradiated skin change in C57BL mice. Phytother. Res. 22, 1423–1427.

Kimura, Y., Sumiyoshi, M., Kawahira, K., Sakanaka, M., 2006. Effects of ginseng saponins isolated from Red Ginseng roots on burn wound healing in mice. Br. J. Pharmacol. 148, 860–870.

Lee, E.H., Cho, S.Y., Kim, S.J., Shin, E.S., Chang, H.K., Kim, D.H., Yeom, M.H., Woe, K.S., Lee, J., Sim, Y.C., Lee, T.R., 2003. Ginsenoside F_1 protects huma HaCat keratinocytes from ultraviolet-B-induced apoptosis by maintaining constant levels of Bcl-2. J. Invest. Dermatol. 121, 607–613.

Lee, J., Jung, E., Lee, J., Huh, S., Kim, J., Park, M., So, J., Ham, Y., Jung, K., Hyun, C.G., Kim, Y.S., Park, D., 2007. *Panax ginseng* induces human Type I collagen synthesis through activation of Smad signaling. J. Ethnopharmacol. 109, 29–34.

Nag, S.A., Qin, J.J., Wang, W., Wang, M.H., Wang, H., Zhang, R., 2012. Ginsenosides as anticancer agents: *in vitro* and *in vivo* activities, structure-activity relationship, and molecular mechanisms of action. Front. Pharmacol. 3, 1–18.

Paik, N.H., Park, M.K., Choi, K.J., Cho, Y.H., 1982. Isolation of ginsneosides Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1 from ginseng root by high performance liquid chromatography. Arch. Pharm. Res. 55, 7–12.

Peyssonnaux, C., Eychène, A., 2001. The Raf/MEK/ERK pathway, new concepts of activation. Biol. Cell 93, 53–62.

Son, J.W., K.H., Oh, D.K., 2008. Ginsenoside Rd production from the major ginsenoside Rb_1 by β-glucosidase from *Thermus caldophilus*. Biotechnol. Lett. 30, 713–716.

Stork, P.J.S., Schmitt, J.M., 2002. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol. 12, 258–266.

Sung, S.H., Park, S.H., Song, S.Y., Lee, S.J., Lee, H.W., Kim, S.H., Lee, M.A., Yoon, I.S., Kim, D.D., Kang, S., Sung, J.H., 2011. Epidermal regeneration by ent-16α, 17-dihydroxy-kauran-19-oic acid isolated from *Siegesbeckia pubescens*. Cell Prolif. 44, 527–536.

Wada, K., Nakajima, A., Katayama, K., Kudo, C., Shibuya, A., Kubota, N., Terauchi, Y., Tachibana, M., Miyoshi, H., Kamisaki, Y., Mayumi, T., Kadowaki, T., Blumberg, R.S., 2006. Peroxisome proliferator-activated receptor g-mediated regulation of neural stem cell proliferation and differentiation. J. Biol. Chem. 281, 12673–12681.

Xue, J.F., Liu, Z.J., Hu, J.F., Chen, H., Zhang, J.T., Chen, N.H., 2006. Ginsenoside Rb_1 promotes neurotransmitter release by modulating phosphorylation of synapsins through a cAMP-dependent protein kinase pathway. Brain Res. 1106, 91–98.

Yuan, H.D., do, Kim, Quan, Y., Kim, H.Y., Jung, S.J., Chung, S.H., M.S., 2012. Ginsenoside Rg_2 induces orphan nuclear receptor SHP gene expression and inactivates GSK3beta via AMP-activated protein kinase to inhibit hepatic glucose production in HepG2 cells. Chem. Biol. Interact. 195, 35–42.